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Establishment of a Counter-selectable Markerless Mutagenesis System in *Veillonella atypica*

Peng Zhou, **Xiaoli Li**, and **Fengxia Qi**[#]

Department of Oral Biology, College of Dentistry, The University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104

Abstract

Using an alternative sigma factor *ecf3* as target, we successfully established the first markerless mutagenesis system in the *Veillonella* genus. This system will be a valuable tool for mutagenesis of multiple genes for gene function analysis as well as for gene regulation studies in *Veillonella*.

Keywords

Markerless; Veillonellae; Mutagenesis; PheS

Veillonellae are one of the most prevalent and numerically dominant bacteria in the oral microbiota (Dzink, *et al.*, 1989, Kamma, *et al.*, 1995, Palmer, *et al.*, 2006, Zaura, *et al.*, 2009, Periasamy & Kolenbrander, 2010). Two characteristics of the *Veillonella* genus make them one of the bridging species in the development of the oral biofilm. One is their utilization of lactate as a preferred carbon and energy source (Rogosa, 1964); the other is their prolific coaggregation with many initial, middle, and late colonizers (Hughes, *et al.*, 1988, Palmer, *et al.*, 2006, Chalmers, *et al.*, 2008). In addition to the human oral cavity, veillonellae are also dominant colonizers of the human gastrointestinal and respiratory tracts (Madan, *et al.*, 2012).

The genus *Veillonella* consists of 13 species (Aujoulat, *et al.*, 2014). Despite their prevalence in the human microbiome, little is known about their biology and pathogenic potential, partially due to our inability to genetically manipulate this group of bacteria until recently. Our group successfully established the first tractable genetic transformation system in a clinical strain of *Veillonella atypica* (Liu, *et al.*, 2012). Using this system, we have made insertional (single-crossover) mutations of several genes important for cell-cell coaggregation (Zhou, *et al.*, 2014). However, the single-crossover mutagenesis system could not create mutations in a multigene operon without posing polar effects on downstream

[#]Corresponding author. University of Oklahoma Health Sciences Center, 975 NE 10th Street, Oklahoma City, OK 73104. Phone: (405) 271-3668. FAX: (405) 271-3603. Felicia-qi@ouhsc.edu.

genes. As our attempts to develop a double-crossover mutagenesis system failed, we sought to develop a markerless mutagenesis system using a single-crossover strategy.

To develop this system, we chose a mutant *pheS* gene as the counter-selectable marker and an alternative sigma factor *ecf3* as the target gene. The *pheS* gene encodes the highly conserved phenylalanyl-tRNA synthetase alpha subunit (Plumbridge & Springer, 1980). A point mutation in *pheS* generating an A294G substitution in *E. coli* confers sensitivity to the phenylalanine analog *p*-chloro-phenylalanine (*p*-Cl-Phe), and has been used as a counter-selectable marker to create markerless mutations in several bacterial species (Kast & Hennecke, 1991, Kristich, *et al.*, 2007, Barrett, *et al.*, 2008, Xie, *et al.*, 2011, Carr, *et al.*, 2015).

To create the mutant *pheS* in *Veillonella*, a GCC → GGT mutation was created from the OK5 *pheS* gene, generating an A308G (equivalent to A294G in *E. coli*) substitution. First, the wild-type *pheS* was amplified by PCR using primers *pheS*-F and *pheS*-R-*Eco*RI (Table 1). Next, the highly expressed OK5 *mdh* (malate dehydrogenase) promoter was amplified by PCR using primers P*mdh*-F-*Xho*I and P*mdh*-R, and fused with *pheS* by overlapping PCR. The PCR amplicon was double digested with *Eco*RI and *Xho*I and inserted into the suicide vector pBST containing the tetracycline resistance gene *tetM* (Liu, *et al.*, 2012). The recombinant plasmid pBST-P*mdh-pheS* was then used as template for inverse PCR using the phosphorylated primers *pheS*m-F and *pheS*m-R, which contained the site-specific mutation (GCC → GGT), to create *pheS**. The PCR product was ligated and transformed into *E. coli* DH5α. Plasmid pBST-P*mdh-pheS** containing the expected GCC → GGT mutation was confirmed by sequencing. This plasmid was later used as a carrier for the markerless deletion of the target gene.

To delete complete *ecf3* ORF, the upstream and downstream regions were amplified by PCR using primer pairs *ecf3*-KO-up-F/ *ecf3*-KO-up-R and *ecf3*-KO-down-F/ *ecf3*-KO-down-R, respectively (Table 1). The two PCR amplicons were then ligated by overlapping PCR. The PCR product was double digested with *Xba*I and *Bam*HI and ligated with plasmid pBST-P*mdh-pheS**. The recombinant plasmid pBST-P*mdh-pheS***-ecf3* was confirmed by PCR and sequencing.

The *ecf3* markerless deletion strain Δ*ecf3* was constructed by a two-step process: single-crossover integration, and recombinative excision. First, plasmid pBST-P*mdh-pheS***-ecf3* was transformed into *V. atypica* OK5 via electroporation as previously described (Liu, *et al.*, 2012). The transformation mixture was plated on brain-heart-infusion plus 0.6% lactate (BHIL) plates containing 2.5 μg ml^{-1} tetracycline (Tet). Tet resistant colonies all contain the transforming plasmid integrated either at the upstream or downstream regions of *ecf3* via single-crossover recombination (Fig. 1A). Positive colonies were further purified and confirmed by PCR (data not shown).

For the second step, a randomly selected positive clone was grown overnight in liquid BHIL without antibiotics to allow recombinative excision of the plasmid. The overnight culture was then serially diluted and plated on BHIL plates containing 15 mM *p*-Cl-Phe for counter-selection. Cells growing on this plate all lost the plasmid via recombinative excision (Fig.

1A). These cells also automatically lost resistance to tetracycline. As recombinative excision can occur with equal chances at the upstream or downstream regions of *ecf3*, theoretically ~50% of *p*-Cl-Phe resistant, Tet-sensitive colonies should contain the deletion while the other 50% should recreate a wild-type genotype (Fig. 1A).

To determine which colony contained the *ecf3* deletion, chromosomal DNA was isolated from randomly selected colonies, and the primer pair *ecf3*-KO-up-F/*ecf3*-KO-down-R (Table 1) was used to amplify the *ecf3* surrounding regions by PCR. The wild-type genomic DNA was used as a control. As demonstrated in Fig. 1B, a 3-kb PCR band was obtained from the wild-type, while a 2.2 kb fragment was generated from one of the mutants. To confirm the 2.2 kb PCR product indeed contained the expected deletion, the DNA band was sequenced and showed the correct deletion (data not shown).

Among 20 colonies thus tested, 7 have the expected deletion, while 13 have the wild-type genome type (data not shown). While this difference may not be statistically significant due to the small sample size, this slightly lower percentage of deletion mutant could be due to the longer upstream region (1300 bp) vs the downstream region (1000 bp) used in constructing the *ecf3* deletion. As illustrated in Fig. 1A, a longer upstream fragment could increase the chance of recombination at this region over the shorter downstream region.

In summary, we have successfully created the first markerless mutagenesis system in *V. atypica*. Combined with the single-crossover mutagenesis and the shuttle plasmid system constructed previously (Liu, *et al.*, 2012), we now have a versatile genetic tool box, which can be used not only for gene deletion studies, but also for insertion of reporters, because the same principle illustrated in Fig. 1A also works for insertions. This work was supported by an NIH/NIDCR grant 2R15DE019940 to FQ.

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Highlights

The First markerless system in *Veillonella*

A Versatile mutagenesis system

Counter-selection

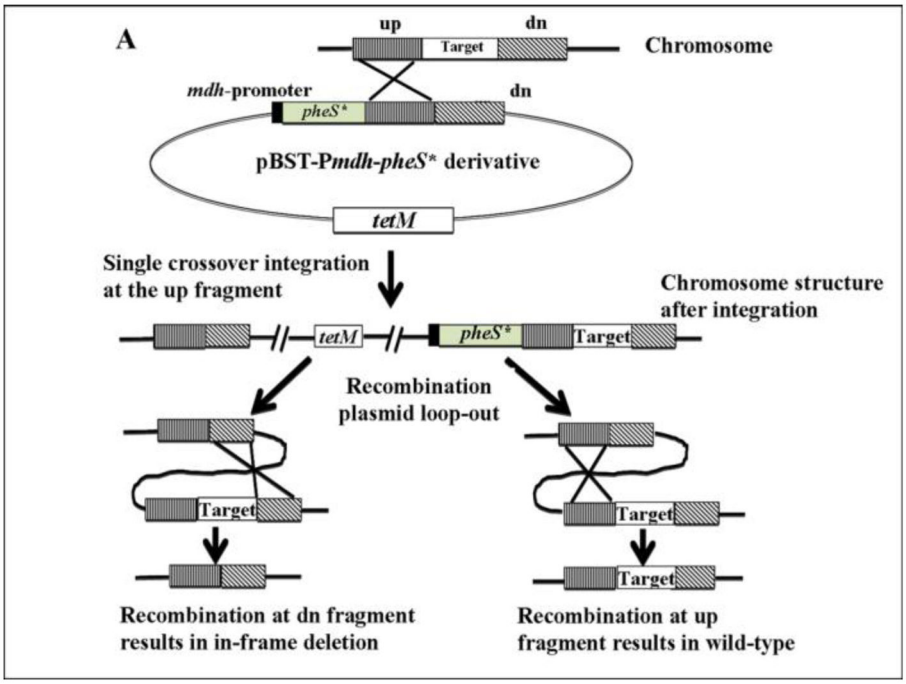

Fig. 1A

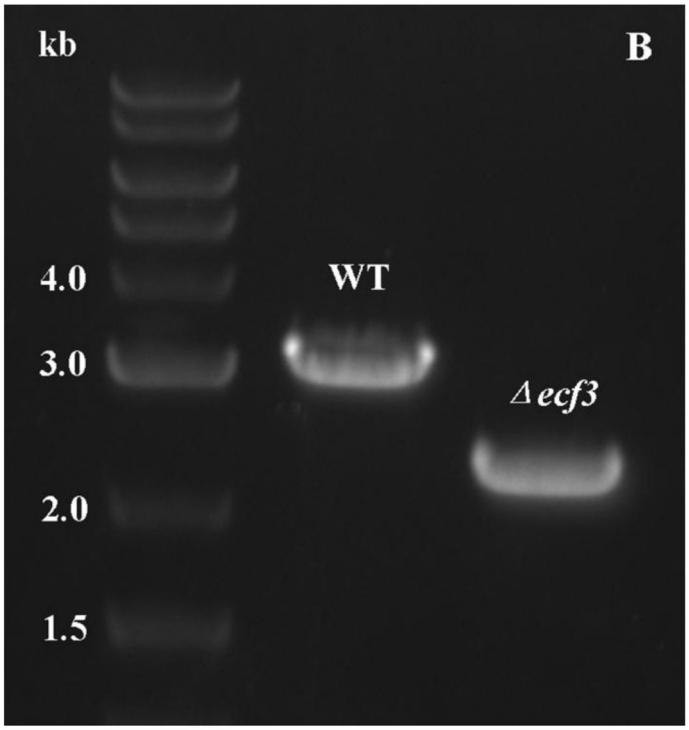

Fig. 1B

Fig. 1.

Construction of a *pheS*-based markerless mutagenesis system in *V. atypica*. (A), a schematic presentation of the strategy for constructing the markerless deletion system. Here only integration at the upstream region is illustrated. Integration can also happen at an equal chance in the downstream region. When this happens, the result from the second step is opposite to what illustrated here; i. e. recombination excision at the downstream region would recreate the wild-type genotype, while recombination at the upstream region would generate the deletion. (B), confirmation of Δ*ecf3* deletion by PCR. The expected wild-type amplicon is approximately 3.0 kb, while the Δ*ecf3* deletion mutant is expected to be approximately 2.2 kb. Only one clone is shown here.

Table 1

Bacterial strains, plasmids and primers used in this study

	Characteristics	**Reference**
Strains		
E. coli DH5α	Cloning strain	
V. atypica OK5	Wild type	(Liu *et al*., 2012)
Δ*ecf3*	OK5 *ecf3* deletion mutant	This work
Plasmids		
pBST	Suicide vector of *V. atypica*, the beta-lactamase gene in pBluescript II KS (+) was replaced by *tetM*	(Liu *et al*., 2012)
pBST-P*mdh-pheS**	Carrier plasmid for markerless deletion of any gene	This work
pBST-P*mdh-pheS*-ecf3*	Carrier plasmid for *ecf3* markerless deletion	This work
Primers	Sequence (5′ to 3′)	Purpose
pheS-F	ATGGAACAAGAATTACAACGCATA	*pheS* amplification
pheS-R-*Eco*RI	CGGAATTCCTAAAATTGTTCCAAGAAACGGATATCA	*pheS* amplification
P*mdh*-F-*Xho*I	CCGCTCGAGATACATACATCACTATATCTGTAACA	*mdh* promoter amplification
P*mdh*-R	TATGCGTTGTAATTCTTGTTCCATTGTTAAAACCTCTTTTCAGAAAATATGTA	*mdh* promoter amplification
*pheS*m-F	CCAAAACCTTTCACCTTATTAGGATCA	Site-directed mutation of *pheS*
*pheS*m-R	TTTTGGTATGGGCGTAGAACGTA	Site-directed mutation of *pheS*
ecf3-KO-up-F	CGGGATCCGAAAAGAGTTTTTTGTGTGA	*ecf3* deletion
ecf3-KO-up-R	TAAAAAATATTTTAGATTTTTAAAAGATTCGTTCCTTTCTGCCTA	*ecf3* deletion
ecf3-KO-down-F	TAGGCAGAAAGGAACGAATCTTTTAAAAATCTAAAATATTTTTTA	*ecf3* deletion
ecf3-KO-down-R	GCTCTAGAGTATGCCGATATTATAGGCTGCA	*ecf3* deletion